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Maternal yolk androgens in European starlings: affected by social environment or individual traits of the mother?

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Summary

Social competition among female birds has been shown to positively affect yolk androgen levels, perhaps providing a mechanism to communicate environmental conditions to offspring. Whether this relationship is due to social density or to differences among mothers that breed in different social situations is unclear. We manipulated breeding density to test these alternative explanations. Yolk androgens were measured in clutches of European starlings, *Sturnus vulgaris*, breeding in consecutive years in outdoor aviaries of different sizes and with varying numbers of breeding pairs. Testosterone (T) levels increased significantly with increasing density. Dihydrotestosterone (DHT) levels increased with the number of nest boxes available. The number of nest boxes monopolized by a breeding male negatively affected androstenedione (A4) levels, positively affected DHT levels and did not affect T levels. Other factors related to social interactions or competition among females (including polygyny) did not influence yolk hormone levels. Within-female yolk androgen levels were highly consistent over two consecutive years even though females were breeding in opposite breeding densities during each year, suggesting that individual characteristics are important determinants of variation in maternal androgen allocation in addition to potential effects of environmental conditions. This within-female consistency of yolk androgen levels across years has important implications.

Keywords: maternal effects, yolk testosterone, breeding density and social competition, polygyny, repeatability.

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Introduction

Mothers may enhance their fitness considerably by adjusting the development of their offspring to the conditions they will encounter after birth or hatching (Mousseau & Fox, 1998). One of the pathways for such maternal effects that currently attract a lot of interest is via hormone-mediated maternal effects. This is because steroid hormones from maternal origin reach the developing embryo in many animal taxa, exerting short and long term influences on their phenotype (reviewed by Groothuis et al., 2005). The embryo's exposure to maternal hormones is likely a function of the hormonal state of the mother, which in turn is depending on environmental conditions. Therefore, hormone-mediated maternal effects offer an excellent pathway for adjusting the offspring to current environmental conditions that the mother is experiencing during reproduction.

Androgens are of special interest here. They play an important role in territorial interactions during reproduction, which are stimulated by increased population density, which in turn may lead to increased levels of androgen production (e.g., Wingfield et al., 1990; Nelson, 1995; Silverin, 1998; Møller et al., 2005; but see Van Duyse et al., 2004). In addition, increased levels of androgens in turn enhance dominance and aggressiveness (e.g., Searcy & Wingfield, 1980; Wingfield et al., 1990). Although levels of androgens are usually lower in female than in male vertebrates, female levels are also known to fluctuate in relation to environmental conditions (e.g., Chapman et al., 1998; Smith et al., 2005; see Staub & de Beer, 1997 for a review on the role of androgens in female vertebrates and for bird species in particular Ketterson et al., 2005). Hormone-mediated maternal effects can be relatively easily studied in birds, where most of the offspring development occurs outside the mother's body in a sealed environment. Schwabl (1993) has shown that avian egg yolks are indeed a source of maternal hormones and several studies have shown that these maternal hormones can have profound short- and long-term effects on the offspring's phenotype (e.g., Eising et al., 2001; Eising & Groothuis, 2003; for a review see Groothuis et al., 2005).

Among the most studied factors that determine among clutch variation in yolk androgens of maternal origin in bird species is social density. For example, in House sparrows *Passer domesticus* social density was positively correlated with yolk T levels (Schwabl, 1997) and both maternal and yolk T levels increased with increasing number of occupied neighbouring nest

boxes (Mazuc et al., 2003). In Tree swallows *Tachycineta bicolor*, maternal aggressive response levels to conspecific intrusions at the nest box were reflected in the T levels in the eggs (Whittingham & Schwabl, 2002). In American coots *Fulica americana* (Reed & Vleck, 2001) and Black-headed gulls *Larus ridibundus* yolk androgen levels increased with breeding density, and in gulls these levels also decreased with increasing vegetation height, likely due to a reduced amount of social interactions between breeding individuals (Groothuis & Schwabl, 2002; Groothuis et al., 2005).

Also in our own study species, the European starling, *Sturnus vulgaris*, Pilz & Smith (2004) have shown that yolk androgen levels increase with increasing breeding density. This relationship may be mediated by higher levels of inter-female aggression, in particular towards floater females searching for mates or opportunities to parasitize broods in large colonies. European starlings are facultatively polygynous (Pinxten et al., 1989). Since female fitness may decrease with loss of paternal investment when floaters settle as secondary females (Pinxten & Eens, 1990, 1994; Eens & Pinxten, 1996), females aggressively defend their territories and mating status against female conspecifics (Pinxten & Eens, 1997; Sandell & Smith, 1997). The proportion of time spent on aggressive behaviours is highest during the pre-laying period (see Pavlova et al., 2007), i.e., during the time of yolk formation and, thus, likely to be reflected in yolk androgen levels.

All previous studies have used a correlative approach to determine the relation between breeding densities and yolk hormone levels. In this way one cannot exclude the possibility that the observed patterns are merely a reflection of differences in maternal phenotype rather than an effect of social density per se. For example, if aggressive females choose or are able to defend a breeding site in a high social density and at the same time allocate higher hormone levels to their eggs, the correlation between environment and yolk hormone levels may actually be determined by the mother's phenotype rather than the environment itself.

To disentangle these two possibilities we studied the effect of social density by manipulating the latter. Based on the data presented by Pilz & Smith (2004), our experimental design allowed us to study allocation patterns of maternal yolk androgens in captive European starlings breeding in outdoor aviaries in relation to breeding densities and nest site availability in two subsequent years. We hypothesize that yolk androgen levels increase with increasing breeding density and with increasing competition for nest boxes

of the same polygynous male. In addition, we reversed the breeding density treatment of individual females between subsequent years allowing for a sensitive within female comparison and the analysis of individual consistency in yolk androgen deposition. To the best of our knowledge, such within-female consistency of yolk androgen levels across years has never been examined.

Methods

Study species and housing conditions

European starlings are hole-nesting songbirds that show normal reproductive behaviour in captive conditions (Eens et al., 1990, 1993). The experiment was carried out in eight different outdoor aviaries (see Table 1) situated at the campus of the University of Antwerp, Belgium. All aviaries were provided with identical nest boxes (with an inter-nest box distance of approximately 1 m), dry nest material, wooden perches and food and water ad libitum. All birds were marked with metal- and colour-bands for identification. Starlings were held in agreement with Flemish and Belgian laws concerning the protection of animal welfare.

Experimental set up

Starlings were kept in single-sex groups during the non-breeding season. In April of both years birds were re-assigned to any of the seven (2002) or eight (2003) experimental aviaries, in mixed-sex groups with approximately equal numbers of males and females. The experimental aviaries differed in surface area, total number of birds allocated to each and in the number of nest boxes potentially available for breeding (see Table 1). Females that bred in low density aviaries in 2002 were housed in high density aviaries in 2003, and vice versa. Females that were used in both years were housed with different males in both years. All females used in this experiment were at least 3 years old.

Nest box occupation, pair formation and egg laying were monitored daily to determine the occurrence of polygyny and the exact laying date and laying order. Fresh eggs were removed, marked and replaced with dummy eggs, which were removed on the 7th day of incubation. Afterwards, nest boxes were checked again daily to monitor the production of a replacement clutch.

Table 1. Characteristics of each of the eight aviaries involved in the experiment: surface area, number of birds per aviary, total number of eggs produced per aviary and % nest boxes with active nests.

Aviary	AK1	AK2	AA	AM	DCR2	DCL1	DCL2	DCN*
Surface area (m^2)	5	5	10	10	95	98	98	138
No females (breeding) 2002	1 (1)	1 (0)	1 (1)	1 (0)	–	6 (2)	20 (2)	27 (16)
No females (breeding) 2003	1 (0)	1 (1)	1 (1)	1 (1)	3 (3)	6 (6)	20 (5)	22 (0)
Total no birds 2002/3	2/2	2/2	2/2	2/2	–/7	14/14	37/37	52/46
Density (No. of birds – $2/m^2$) 2002/3	0/0	0/0	0/0	0/0	–/0.05	0.12/0.12	0.36/0.36	0.36/0.32
Total no eggs 2002/3	4/0	–/2	3/9	–/8	–/18	6/49	7/21	68/0
No. of nest boxes	2	2	2	2	6	12	10	25
Boxes with active nests 2002/3 (%)	50/50	0/50	50/50	0/50	–/50	17/50	20/50	68/0

* In 2002 five males in this aviary were polygynous.

The latter was also removed and replaced by dummy eggs till day 7 of incubation. Eggs were weighed to the nearest 0.01 g and then stored at −20°C until the hormone analyses were performed. A total of 22 females produced 88 eggs in 2002 while in 2003 17 females produced 107 eggs (Table 1). Surprisingly, none of the adult females housed in DCN in 2003 started laying. A total of 193 eggs (2 eggs were broken) of 57 clutches (including 18 replacement clutches) were assayed. In total 15 females produced at least one clutch in each of the two study years. Within the largest aviary (DCN) 5 males were polygynous and bred with 11 different females.

Hormone analyses

A competitive-binding radioimmunoassay (RIA) was used to determine androstenedione (A4), dihydrotestosterone (DHT) and testosterone (T) levels in the egg yolks. Radioimmunoassays (RIAs) followed the protocol as described by Wingfield & Farner (1975) and Schwabl (1993), with slight modifications. Yolks were weighed and homogenized with equal (1 ml/g yolk) amounts of water. Approximately 150 mg of this mixture was taken and enriched with 2000 cpm of tritiated A4, DHT and T (NEN, The Netherlands) for calculation of recoveries. Samples were left to equilibrate overnight and afterwards extracted using 2 × 4 ml diethyl-/petroleum ether (70:30, v/v), dried under nitrogen and reconstituted in 1 ml 90% ethanol. Samples were

stored in the freezer at −20°C for at least 12 h and then spun down to remove neutral lipids. Extracts were dried again and reconstituted in 1 ml 2% ethylacetate in 2,2,4-trimethylpentane before they were transferred to diatomaceous earth chromatography columns to elute each hormone fraction. Respective concentrations of 2%, 10% and 20% ethylacetate were used to wash out A4, DHT and T fractions. Using a competitive binding RIA with hormone specific antibodies (Endocrine Science, USA), hormone concentrations (pg steroid per mg yolk) were determined in five double radioimmunoassays over a period of two years.

The average recovery rate for A4 was 55.5%, 27.1% for DHT and 37.5% for T. These recovery rates are relatively low, but still similar to those published in other studies (e.g., Reed & Vleck, 2001; Pilz et al., 2005). Since average hormone levels and the pattern over the laying sequence was similar as in other studies (see Results) we are confident that the data are meaningful. Data which showed less than 20% recovery rate or intra-assay variation over 15% were excluded from the analyses ($N = 3, 9, 13$ for A4, DHT and T, respectively). The intra-assay variation coefficients were for A4, DHT and T 5.34%, 5.13% and 5.62%, respectively. Unfortunately, inter-assay coefficients of variation were very high (25.2% for A4, 54.4% for DHT and 39.9% for T; see below), perhaps due to the fact that assays were performed in different years, using different batches of chemicals. The high inter-assay variation is unlikely to be random as intra-assay coefficients of variation were low.

Statistical analyses

Log-transformation successfully normalized A4 and T levels (Kolmogorov–Smirnov test; $p = 0.46$ and $p = 0.35$, respectively). Untransformed DHT levels were statistically normally distributed with the exclusion of one outlier ($\Delta > 5$ s.d.; $p = 0.06$). Hormone concentrations varied markedly between assays, years and aviaries, while the latter three were highly significantly inter-correlated (all $p < 0.001$), potentially obscuring more interesting effects of social density parameters. Using assay as a random factor in all models left the effects of year and aviary redundant (all $p > 0.17$). Assay effects were significant throughout (all $p < 0.05$) unless otherwise indicated and have not been included in the tables of effects in the text. Other effects of

clutch characteristics (clutch number (within a year), clutch size, laying order, laying date, egg mass and yolk mass) and factors related to social density (density (No. of birds – $2/m^2$), total number of available nest boxes and number available per female through her partner, polygyny of the partner, the number and proportion of nest boxes with active nests (at any stage of the breeding cycle), and their potential interaction effects on hormone levels were analysed using Linear Mixed models in MLWin (Rasbash et al., 2000). For the calculation of density, the number of birds per aviary to interact with other than the female itself and her breeding partner was taken (No. of birds – 2).

Females were added as random factors to the model, with eggs nested within a clutch and clutches nested within each female. Thus, also the fact that some females produced more than one clutch per year or bred in both years has been taken into account. Biologically meaningful models were built by stepwise forward adding of non-correlated explanatory variables. Final Wald tests for fixed effects were performed by dropping individual terms from the full fixed model and only significant factors were retained. Wald statistics (W), which follow a χ^2-distribution, degrees of freedom and p values are presented in tables or text. In instances where correlated factors were both significant in similar models, both are presented in the text. The statistical package SPSS was used to test for normality of the data, Pearson's correlations between explaining variables and for performing matched sample T-tests on the females that bred in different densities in subsequent years. Repeatability of hormone levels of individual females was calculated according to Lessells & Boag (1987).

Results

We found average clutch levels of 43.61 $\pm$ 12.46 ng/yolk A4 ($N = 56$ complete clutches), 6.47 $\pm$ 0.54 ng/yolk DHT ($N = 50$) and 0.96 $\pm$ 0.10 ng/yolk T ($N = 48$). Within females, yolk hormone levels were significantly inter-correlated. Log A4 levels varied negatively with log DHT levels ($c = -0.32$, $p = 0.006$, $N = 182$), positively with log T levels ($c = 0.16$, $p = 0.03$, $N = 177$) and log DHT levels varied positively with log T levels ($c = 0.28$, $p < 0.001$, $N = 172$).

Laying order and density dependent effects

The most parsimonious model explaining log-transformed A4 levels contained three relevant variables (Table 2, Log A4). Log A4 levels increased with laying order, decreased with laying order squared and decreased with egg mass (Table 2, Log A4; Figure 1A). None of the other variables related to bird density in the aviary affected A4 levels. However, one alternative significant model was found when the number of nest boxes monopolized by a female's male partner was included (negative effect, Table 2). These two alternative models cannot be compared directly as the dataset for the second model is reduced due to the fact that it would be meaningless to incorporate here the aviaries in which only one breeding pair is present.

Log T levels increased with laying order, decreased with laying order squared, decreased with egg mass (Figure 1B) and increased with bird density in an aviary (Table 2, Log T; Figure 2). Other attributes of the aviary or a female's breeding partner did not affect the T levels in their eggs.

DHT levels increased with laying order or laying order squared, (Figure 1C) but in none of the models did these effects become significant (Table 2, DHT). Therefore, laying order was excluded from further analyses. Eggs mass negatively affected yolk DHT levels in all models (Table 2). In addition, clutch number affected DHT, with levels increasing in subsequent clutches. Both factors were retained in the final model (egg mass did not vary with clutch number, $W = 2.12$, $p = 0.12$). Finally, there was a significant positive effect of the total number of nest boxes in an aviary (Figure 3). Substitution of the number of nest boxes by the number monopolized by each male (which again reduces sample size) showed a similar significant positive effect (Table 2, DHT). In this model clutch number did not contribute significantly ($W = 2.43$, $p = 0.09$) and was eliminated. Other substitutions of density related parameters did not yield significant results.

Thus, for all three hormones there were indications of an increase with laying order and a decrease with egg mass (despite the fact that egg mass decreased significantly with laying order; $W = 10.51$, $p = 0.001$). Social density affected T concentrations, whereas the total number of nest boxes available in an aviary influenced yolk DHT levels. Finally, the number of nest boxes occupied by a male varied negatively, but weakly, with A4 concentrations and strongly and positively with DHT concentrations.

Table 2. Outcomes of the tested Linear Mixed Models for density dependant effects for androstenedione (A4), testosterone (T) and dihydrotestosterone (DHT). Presented are parameter estimates and standard errors, degrees of freedom, Wald statistics for fixed effects, p values and Aikaike's Information Criterion ($-2*$Log-Likelihood $+ 2*K$, where K is the number of estimable parameters) used for best model selection. It should be noted that in these tables Final models do not necessarily represent the best models as often the differences between models were very small or models could not be directly compared due to a difference in sample size.

		Estimate	SE	df	W	p	AIC
Log A4							
Null model							
Fixed	Constant	0.91	0.07	1			
Deviance		152.06		181			168.06
Basic model							
Fixed	Constant	0.65	0.12	1			
	Lay order	0.22	0.10	1	6.72	0.010	
	Lay order^2	−0.04	0.02	1	5.07	0.024	
Deviance		144.14		179			164.14
Final model 1							
Fixed	Constant	1.13	0.04	1			
	Lay order	0.21	0.08	1	6.25	0.012	
	Lay order^2	−0.04	0.02	1	5.14	0.023	
	Egg mass	−0.07	0.04	1	2.66	0.103	
Deviance		140.57		177			**162.57**
Final model 2							
	Constant	1.54	0.27	1			
	Lay order	0.18	0.09	1	4.30	0.038	
	Lay order^2	−0.03	0.02	1	3.65	0.056	
	Egg mass	−0.09	0.04	1	4.49	0.034	
	Box per male	−0.10	0.04	1	4.80	0.028	
Deviance		112.05		155			136.05
Log T							
Null model							
Fixed	Constant	−0.29	0.04	1			
Deviance		−52.25		171			−36.25
Basic model							
Fixed	Constant	−0.60	0.07	1			
	Lay order	0.21	0.04	1	23.81	<0.001	
	Lay order^2	−0.03	0.01	1	11.19	<0.001	
Deviance		−102.08		169			−82.08

Table 2. (Continued).

		Estimate	SE	df	*W*	*p*	AIC
Final model							
Fixed	Constant	−0.30	0.18	1			
	Lay order	0.20	0.04	1	21.64	<0.001	
	Lay order^2	−0.03	0.01	1	10.82	0.001	
	Egg mass	−0.06	0.03	1	5.39	<0.020	
	Density	0.33	0.15	1	4.79	0.029	
Deviance		−110.99		167			**−86.99**
DHT							
Null model							
Fixed	Constant	1.80	0.99	1			
Deviance		1006.01		184			1022.01
Basic model							
Fixed	Constant	1.76	0.84	1			
	Lay order	0.21	0.18	1	1.50	0.220	
	Lay order^2	0.03	0.03	1	0.85	0.360	
Deviance		1004.48 or 1005.14		183			1022.48 or 1023.14
Final model 1							
Fixed	Constant	7.57	2.81	1			
	Egg mass	−0.86	0.40	1	4.84	0.028	
	No. of boxes	0.13	0.07	1	3.91	0.048	
Clutch nr		1.87	0.73	1	6.68	0.010	
Deviance		993.16		181			**1015.16**
Final model 2							
Fixed	Constant	8.35	3.06	1			
	Egg mass	−0.70	0.41	1	2.83	0.090	
	Box per male	1.54	0.44	1	12.16	<0.001	
Deviance		871.70		158			891.70

Effects of polygyny

Beside density related competition, competition between females paired to the same male could potentially influence yolk hormone levels. In the largest aviary in 2002, 16 females were paired to 10 males. Five males were polygynous and 11 females were paired with these males. Twice two females paired with the same male also bred simultaneously (laying of first egg within 4 days of each other), potentially increasing the level of competition between these females. Therefore, within this particular aviary we also tested whether such competition influenced yolk hormone levels.

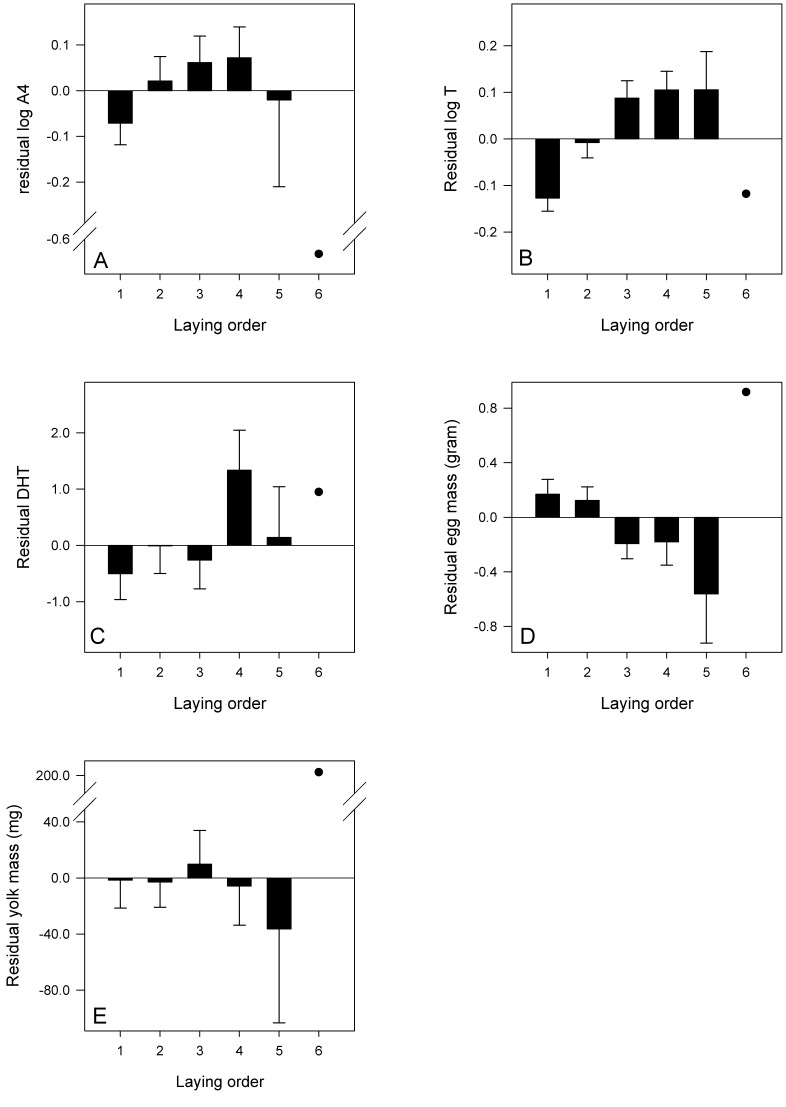

Figure 1. Residual yolk androgen content (androstenedione (A4), testosterone (T) and dihydrotestosterone (DHT) (A-1C), egg mass (D) and yolk mass (E) over laying order in adult females ($N = 23$). Black circles indicate a single data point. Presented are the residuals for each dependent variable of the Linear Mixed model which included the random nested design and for the hormone data, assay effects for that variable.

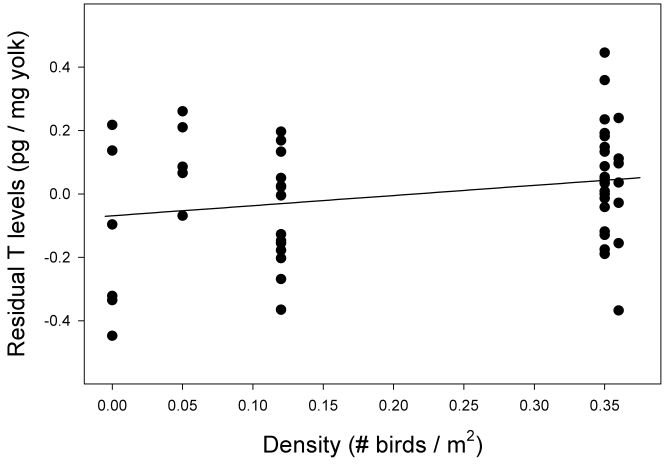

Figure 2. Residual clutch testosterone (T) levels in relation to density within the aviary ($N = 57$ clutches, including 18 replacement clutches). Residuals were calculated from the full Linear Mixed model, which included the random nested design, assay, laying order, laying order squared and egg mass. Residuals were averaged per clutch for visual purposes only. The line represents the regression line.

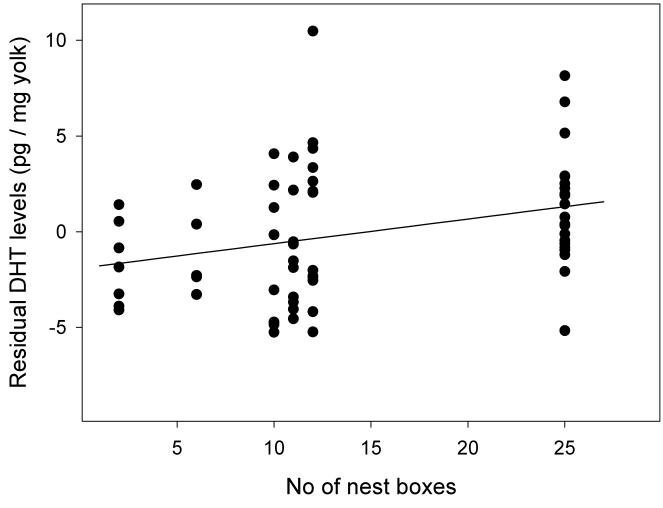

Figure 3. Residual clutch dihydrotestosterone (DHT) levels in relation to the total number of nest boxes available in the aviary ($N = 57$ clutches, including 18 replacement clutches). Residuals were calculated from the full Linear Mixed model, which included the random nested design, assay, clutch number and egg mass. Residuals were averaged per clutch for visual purposes only. The line represents the regression line.

For this sub-sample ($N = 68$ eggs), assay did not have an effect on any of the three hormones (all $p > 0.48$) and was excluded from all analyses. In addition, the patterns of laying order, laying order squared and nest box availability per female were similar to previously found in the large dataset, but were not significant for log A4 levels (all $p > 0.13$). Also being paired to a polygynous male, male identity, pairing date or breeding simultaneously with another female paired to the same male did not significantly influence yolk A4 levels (all $p > 0.25$). The strongest effect was a negative trend for egg mass ($W = 3.72$, $p = 0.054$).

Log transformed T levels were only significantly influenced by laying order ($W = 17.96$, $p < 0.001$). None of the other factors such as mating system, male identity, simultaneous breeding, nest box ratio or pair date contributed to the model (all $p > 0.20$).

Finally, the most parsimonious model explaining DHT level variation contained an effect of egg mass (Estimate $= -1.02$, $W = 2.83$, $p = 0.09$) and of male identity ($W = 25.01$, df $= 10$, $p = 0.005$). There was a non-significant positive trend for polygyny (Estimate $= 2.26$, $W = 2.89$, $p = 0.09$) when substituted for male identity, indicating DHT levels were higher in eggs of females paired to polygynous males.

Individual consistency of yolk androgen levels

Fifteen females bred in both years; 3 females bred first in the highest density and subsequently in isolation (see methods), 8 females went to a lower density in the second year (0.35-0.12 or 0.05 birds/m^2) and 2 females from isolation and 2 from low density (0.12 birds/m^2) went to high density (0.36 birds/m^2) in the next year. In order to do a matched pair comparison, we considered only the first laid egg of the first laid clutch in both years (corrected for assay effects). Within the group of eleven females that went to lower density situations, none of the hormone concentrations was significantly affected by the altered density (paired-sample T-test, all $p > 0.45$). Since we had only four females that went from low to high density, we could not test this effect independently. Above results suggest that yolk hormone concentrations are specific to a certain female and likely to be repeatable, regardless of the breeding circumstances. Accordingly, we found that the repeatability for all three hormones was relatively high and significant, for A4 levels 0.43 ($F_{14,15} = 2.54$, $p = 0.042$), for DHT levels 0.46 ($F_{13,14} = 2.72$, $p = 0.037$) and for T levels 0.50 ($F_{11,12} = 2.99$, $p = 0.036$; see Figure 4).

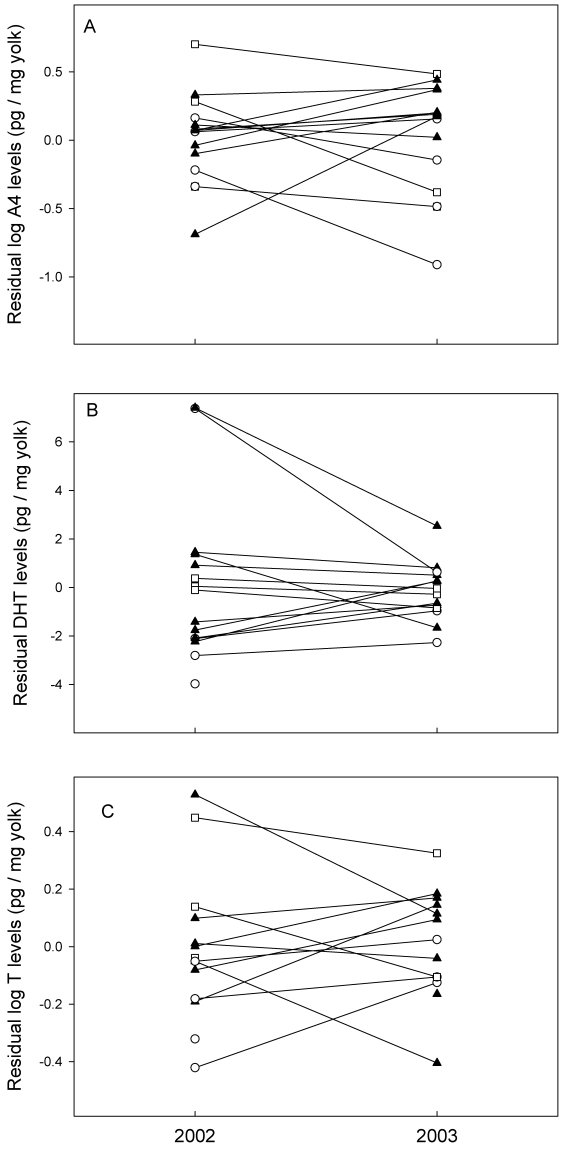

Figure 4. Yolk hormone levels (corrected for assay variation) in first laid eggs of first laid clutches of individual females in two consecutive years (androstenedione (A4) (A); dihydrotestosterone (DHT) (B); testosterone (T) (C)). Females denoted with □ went from high density to isolation in subsequent years, females denoted with ▲ went to a lower density and females indicated with ○ went from isolation to a higher density (for details see text). Sample sizes for the repeat were $N = 15$ for A4, $N = 14$ for DHT and $N = 12$ for T due to missing hormone values.

Discussion

Breeding density has been shown to correlate with yolk hormone levels positively in several species (Schwabl, 1997; Reed & Vleck, 2001; Mazuc et al., 2003) including the European starling (Pilz & Smith, 2004). These studies have been of a correlative nature and could, therefore, not exclude the potential role of maternal phenotype as source of the hormonal variation observed in relation to density dependent effects. We expected to find similar effects in our present study, where we have tried to experimentally induce density and competition-related variation.

Density-dependent effects

Our experimental results tentatively confirm the correlation between social density and yolk T levels reported by Pilz & Smith (2004) as we found that birds that bred at high densities laid eggs containing relatively high T levels. This is consistent with the well known phenomenon that testosterone production is stimulated by social interactions (Wingfield et al., 1990). However, the other androgens were not affected by the number of birds per square meter, perhaps because it does not accurately reflect the level of social competition, due to the fact that not all birds per aviary became reproductively active. Although we found no significant effects of the number of active nest boxes within each aviary, contrary to the correlative study by Pilz & Smith (2001), DHT levels did increase with the total number of nest boxes available. These androgen levels also increased with the number of nest boxes their mate was defending. Some males monopolized up to four nest boxes simultaneously, perhaps serving as an indicator of their individual quality. If females would benefit from being paired to such a high quality male, we could expect competition for these boxes among females to be fierce and androgen levels in their clutches to be high (sensu Gil et al., 1999; von Engelhardt et al., 2004). However, we found that the number of boxes occupied by a male was negatively related to A4 levels and not related to yolk T levels. At present we cannot explain this puzzling effect. Unfortunately we have no detailed behavioural observations, quantifying how many aggressive encounters really took place.

In conclusion, we found some effects of our experimental manipulation of density related parameters on yolk levels of androgens. Yet, as the average hormone levels measured in this study were very similar to values

for starlings reported elsewhere (e.g., Lipar, 2001; Pilz et al., 2003; Pilz & Smith, 2004), we have no reason to suspect that the measurements are inaccurate and, therefore, obscuring strong breeding density effects. However, even though hormone levels in Pilz & Smith (2004) are expressed in a different way and were not related to the same parameters as in our study, the observed effects of social density in both studies are quite comparable in magnitude. Taking the data from Figure 2 in Pilz & Smith (2004) and assuming an average yolk mass of 1 g, Pilz & Smith found testosterone concentrations approximately ranging between 0.55 and 1.4 pg T/mg yolk in relation to the proportion of active nest boxes in a colony, whereas we found a range of 0.8-1.2 pg T/mg yolk in relation to density, although these latter data are uncorrected for assay effects. Our results may have been somewhat confounded by the fact that in our captive situation social relationships were much more fixed than would have been the case in the wild: the captive birds were in constant social contact and were breeding in higher densities and with a smaller inter-nest box distance. Our system was, therefore, potentially more stable, thus obscuring any additional effects of social density and this could also explain the consistency of yolk hormone levels within individual birds (see below). In addition, in the high density aviaries not all birds were breeding in both years, which somewhat limited the sample size for clutches from high density.

On the other hand, in a recent paper, Verboven et al. (2005) found no effect of a significant increase of the number of intra-specific interactions on yolk androgen levels, whereas they did show an effect on other yolk constituents. Moreover, Goymann et al. (2007) have recently shown that the effects of social interactions on circulating plasma androgen levels depend on the breeding system of a species and should be seen separately from seasonal effects on changes in plasma androgens.

Clearly, further studies, both in the wild and under controlled conditions, are needed to reveal the importance of social environment, separate from phylogenetic, seasonal, individual or other effects, as a determinant of yolk androgen levels.

Effects of laying order and polygyny

Similar to findings in many other non-siblicidal species, we found that within clutches, yolk A4 and T concentrations similarly increase with laying order,

supporting the suggestion that yolk androgen levels may have a function in the compensation for negative effects of hatching asynchrony. Differing somewhat from the data described for free living starlings by Pilz et al. (2003) we found that both A4 and T levels slightly decrease at the end of the laying order (see Figure 1A and 1B). In addition, yolk and egg mass decrease too, suggesting that females are unwilling or unable to invest much in their last laid eggs.

Similar to the findings by Pilz et al. (2003) we also found that females who laid larger yolked eggs did not deposit disproportionately more androgens as A4, DHT and T concentrations did not increase with yolk mass. However, contrary to their findings, we found that clutch mass was inversely related to yolk androgen levels. This has also been found in other species, e.g., Black-headed gulls (Groothuis & Schwabl, 2002). Mothers unable to invest many resources in terms of nutritional quality of their eggs may try to compensate for this reduced quality by upping the androgen levels (Groothuis et al., 2005). Although it was originally hypothesized that high quality females producing high quality eggs would be better able to withstand the costs associated with exposure to high androgen levels, several studies now suggest the opposite. Verboven et al. (2003) showed in a feeding experiment in Lesser black-backed gulls *Larus fuscus* that females in good condition allocated less androgens to their eggs. In addition, Rutstein et al. (2005a) showed that diet quality does not necessarily affect yolk androgen allocation per se, but that mothers on a high protein diet decreased testosterone levels with laying order for male eggs, whereas they increased for female eggs. Thus, complex, context-dependent mechanisms may be involved in determining (sex-specific) androgen allocation. In our study total clutch size nor laying date affected yolk androgen levels, perhaps as a consequence of the ad libitum food availability.

There are several strong indications that variation in yolk androgen levels is not only depending on general environmental characteristics, but is rather intricately related to individual characteristics of the breeding partners. For instance, Gil et al. (1999) have shown in zebra finches *Taeniopygia guttata* that females adjust the levels of androgens in their clutch according to quality of the mate with whom they are paired. In addition, both Gil et al. (2004) and Tanvez et al. (2004) have shown that female canaries *Serinus canaria* invested greater concentrations of testosterone in their eggs when exposed to attractive song repertoires than when exposed to unattractive repertoires.

They inferred from this result that song repertoires convey important information about the reproductive value of a given male. However, recent papers by Rutstein et al. (2005b) and Marshall et al. (2005) showed for the same study species that females paired to attractive males or exposed to attractive song did not deposit more in androgen content of their eggs but, in the zebra finch, rather increased egg mass. Moreover, a recent study on House finches *Carpodacus mexicanus* showed that females deposited significantly more androgens into eggs sired by less attractive males, confirming the idea that androgens are rather used as mediators in a compensatory strategy, thus enabling females to improve the quality of young sired by less attractive males (Navara et al., 2006).

In a recently published paper, Gwinner & Schwabl (2005) showed in European starlings, that most maternal steroid levels in yolks were not affected by a monogamous or polygynous mating status, except for A4 levels which were higher in last laid eggs than in first laid eggs in monogamous pairs relative to polygynous pairs. In our study, we found a suggestion that overall DHT levels were higher in eggs of polygynous fathers, independent of laying order. As polygynous males often show a reduction in paternal care (see Magrath & Komdeur, 2003 for a review and Pinxten et al., 1993; Pinxten & Eens, 1994 for the European Starling) this could well reflect a maternal behavioural strategy to compensate for this reduction in care. Why we find this effect only for DHT is unclear. However, the effect of male identity was much stronger, suggesting that other individual attributes rather than mating system alone may play a role.

Within-female consistency of yolk androgen levels

In agreement with many previous studies, our results indicate that there is significant variation in yolk androgen levels among female starlings, even when breeding under the same conditions (see Figure 4). In addition, by collecting clutches of the same females in successive years, we could calculate, for the first time, the within-female consistency of yolk androgen levels across years. Our results show that between-female differences are quite consistent over time and are only to some extent likely to be influenced by environmental challenges. Our repeatability estimates closely resemble the ones found for yolk A4 and T levels in a recent study by Tobler et al. (2007) on Pied flycatchers *Ficedula hypoleuca*. Although their data describe hormone

levels of successive clutches, laid within two weeks of each other during a single breeding season, not across two seasons as in our study, they also showed large inter-female variation in yolk androgen levels and a remarkable consistency within individual females. Such individual consistency can have broad implications for mating and reproductive strategies, and could be linked to studies on avian personalities (Groothuis & Carere, 2005). Both studies strongly suggest that yolk androgen levels may be a characteristic of individual females and that there may be some underlying genetic variation in yolk androgen levels of individual females. In addition, maternal hormone deposition may be adaptively tuned to individual characteristics of a female's mate.

In conclusion, we found both evidence that maternal androgen levels in European starling eggs are a reflection of the social density situation in which females are breeding as well as of individual phenotype. Maternal androgen levels seem to be a repeatable characteristic of individual females. Thus, density related effects as found in several other species are potentially a partial reflection of differences between individual mothers rather than of environmental characteristics.

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